We will lead three class sessions on protein NMR. The goal of these sessions is to introduce the basic principles behind NMR and examine the many ways in which NMR can be used to address questions in biological systems.

Class time will be used to participate in several activities in small groups designed to get you to think about how NMR might be used in different situations.

You will complete worksheets as part of the class activities and these will be handed in and count towards your grade in the class.

For you and your peers to get the most out of this unit, you will need to come to class prepared. For Nov. 7, you should read the attached review article, “The Quiet Renaissance of Protein Nuclear Magnetic Resonance“ which provides an excellent overview of protein NMR.
The Quiet Renaissance of Protein Nuclear Magnetic Resonance

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ABSTRACT: From roughly 1985 through the start of the new millennium, the cutting edge of solution protein nuclear magnetic resonance (NMR) spectroscopy was to a significant extent driven by the aspiration to determine structures. Here we survey recent advances in protein NMR that herald a renaissance in which a number of its most important applications reflect the broad problem-solving capability displayed by this method during its classical era during the 1970s and early 1980s.

Without receivers fitted and kept in order, the air may tingle and thrill with the message, but it will not reach my spirit and consciousness. Mary Slessor, Calabar, ca. 1910

The first nuclear magnetic resonance (NMR) spectrum of a protein, that of ribonuclease A, was reported in 1957 by the lab of Martin Saunders.1 The introduction of radiofrequency-pulsed excitation, signal averaging, and Fourier transform data analysis into NMR spectroscopy in the mid-1960s and the production of the first superconducting magnets a few years later led to dramatic advances in both basic NMR spectroscopy and its applicability to biological systems.2 By the mid-1970s, solution NMR had come to play a prominent role in biochemistry and molecular biophysics as a way of probing biomolecular structure and interactions, as well as providing readouts for various assays. A superb account of this classical one-dimensional (1D) era of biomolecular NMR is found in a 1981 book by Jardetzky and Roberts.3 That the future is difficult to foresee is reflected by an editorial aside in that work: "The hope held out for a complete determination of the structure of proteins and other flexible biomolecules in solution has not materialized and will not do so in the immediate future". By the mid-1980s, Wüthrich and others determined the structures of a number of small proteins.4–6 This enabled the development of two-dimensional NMR methods, early computers and software, innovation, and nascent molecular modeling. A good deal of the focus of NMR during the following 20 years was on the development and application of NMR as a tool for the structural determination of proteins and nucleic acids. During that time period, NMR was extended to more than two dimensions (usually with multiple nuclei involved),7,8 while the tools of preparative molecular biology allowed both routine high-level production and NMR isotopic labeling of numerous previously inaccessible protein and RNA targets. The use of relaxation measurements to illuminate biomolecular motions also advanced dramatically during this time period.

By the year 2000, it became clear that NMR could not keep pace with X-ray crystallography as an approach to routine structural determination for a majority of proteins and nucleic acids, particularly large molecules and complexes. This is despite the development of residual dipolar coupling measurements, pulsed field gradients, pulse shaping, magnets in which 1H NMR frequencies of 1 GHz are approached, exploitation of the TROSY phenomenon, and sophisticated pulse sequences. Even for small proteins, crystallography usually provides a shorter route to structural determination and, frankly, one that is more accessible to nonspecialists than NMR, thanks in part to the admirable efforts of crystallographers to make their field novice-friendly. Moreover, hopes for new technical breakthroughs comparable in broad impact with that of the introduction of the Fourier transform, two-dimensional spectroscopy, or 1H-detected multidimensional heteronuclear methods have not been realized. Of course, in addition to holding its position as the pre-eminent experimental method for studying biomolecular dynamics, NMR continued to occupy an important niche in structural determination of molecules that proved to be recalcitrant to the formation of crystallographic quality.
of well-diffracting crystals. Nevertheless, there has been much soul-searching the past decade about whether NMR represents an approach that is on the decline as a contributor of high-impact results to basic biological and biomedical research.

It is the thesis of this review that, in fact, biomolecular solution NMR is undergoing a quiet renaissance. As in the Renaissance Age of western Europe, which was spurred in part by a renewed appreciation for Classical form and philosophy, recent progress is based partly on a renewed appreciation for the classical applications of NMR, in which total structural determination is only sometimes the goal, and the emphasis is on a wide range of problem-solving applications. And, just as the Renaissance was also driven by transformative humanism and accompanying advances in science and technology, NMR is being transformed by a series of innovations. While no single one of these technical developments may rise to the landmark status of, say, the introduction of the pulsed excitation methods,16 one of these technical innovations is in the process of enhancing and broadening the impact of NMR in biological research. In this paper, we highlight some of these technical developments and offer examples of important applications. The focus is on solution NMR, with the authors noting that recent progress in solid state NMR has been spectacular, but beyond the scope of this review. The emphasis is also on protein-related work, but we hope the reader will seek out impressive recent applications of NMR to nucleic acids and glycans, work that relies on the same technical advances that are summarized here.9–16

THE ABILITY OF NMR TO ACCESS DILUTE SAMPLES HAS BEEN DRAMATICALLY ENHANCED

Since the first NMR experiments more than 50 years ago, there has been an impetus to obtain improved signal-to-noise (S/N) ratios (increased “sensitivity”). Even in the late 1990s, the inherently low sensitivity of NMR spectroscopy dictated long acquisition times and large quantities of sample, typically at least 200 μL of >0.5 mM protein. However, NMR has seen dramatic improvements in sensitivity over the past 15 years. One factor in this development has been the emergence of very high field (>600 MHz 1H frequency) magnets, as NMR sensitivity is proportional to (field strength)3/2. The largest currently available NMR magnet suitable for use in biomolecular NMR is now a 23.4 T magnet (1000 MHz 1H frequency).

The emergence of superior probes for excitation and signal detection has also dramatically improved S/N ratios in biomolecular NMR. Advances have been based on changing probehead/sample sizes and/or chilling key probe components. The sensitivity of an NMR probe is determined by its “quality factor” (Q), with the resulting S/N ratio being directly proportional to Q1/2. The Q factor for a probe is determined by the resonance frequency ω0, the inductance L, and the resistance R of the probe:17

\[ Q = \omega_0 L / R \] (1)

Q can be increased either by increasing inductance or by lowering resistance. The inductance is determined by the size and geometry of the coils, with the easiest way of increasing L being to reduce the size of the probehead. This has been exploited in the development of microcoil probes that, for a fixed concentration, allow improved sensitivity for dramatically reduced sample volumes.18 Decreasing the resistance has been accomplished by the development of “cryogenic probes” in which the probe detection coil and preamplifier are chilled to a very low temperature with helium gas. Cryogenic probes have the added benefit that cooling the preamplifier reduces the thermal noise in the system, allowing for even greater increases in sensitivity.17 Here we outline the capabilities of both microcoil and cryogenic probes and show examples of how they have improved NMR data collection.

Microcoil probes enhance the NMR S/N ratio and allow collection of data on samples with volumes as small as 5 μL and

![Figure 1. 800 MHz 1H–15N TROSY spectra of low concentrations of 45 kDa human arrestin-1 at 308 K in the absence and presence of bicele-associated rhodopsin. (A) 1H–15N TROSY spectrum of 10 μM wild-type 2H- and 15N-labeled arrestin-1 in pH 6.5 buffer. (B) 1H–15N TROSY spectra of 30 μM 2H- and 15N-labeled F85A/F197A-arrestin-1 (black) in the presence of a saturating level (60 μM) of light-activated, phosphorylated rhodopsin (P-Rh*) in bicelles (red) at pH 6.5. Panel B was adapted from ref 25 and used with permission of the publisher. Copyright 2013 National Academy of Sciences.](image-url)
only nanomoles of sample for 15N- and 13C-labeled proteins.\textsuperscript{19,20} The use of microcoil technology also confers two additional advantages besides low sample concentration and small volume. The first is the ability to generate novel pulse sequences that exploit the enhanced radiofrequency power handling of solenoid coils relative to the saddle configuration.\textsuperscript{21} Another capability of microcoil probes is that they can be adapted for flow-through mode for use as an analytical detector in conjunction with liquid chromatography. An example of the use of microcoil probes is provided by NMR measurement of the translational diffusion coefficients of the β2-adrenergic receptor, a G-protein-coupled receptor (GPCR), in a variety of different micelles and mixed micelles.\textsuperscript{22} For these studies, a 1 mm sample diameter microcoil probe was used, for which the sample volume was a mere 6 μL.

The underpinning theory for cryogenic probe technology was presented in the late 1970s by Hoult and Richards.\textsuperscript{23} The first such probe was constructed in 1984.\textsuperscript{24} Widespread access to cryogenic probes became common by the mid-2000s. Commercial cryogenic probes are now typically the “default” probe installed in spectrometers dedicated to biomolecular studies. For any given sample, cryogenic probes allow for a 3–4-fold increase in the S/N ratio relative to that of a same-generation conventional probe. Because NMR experiments are based on averaging of the signals from accumulated scans, and the spectral S/N ratio is proportional to the square root of the number of scans, this 3–4-fold increase in sensitivity corresponds to a 9–16-fold decrease in the time required to achieve a desired S/N ratio.\textsuperscript{17}

Many of the NMR-based advances in biological research during the past decade could not have been accomplished without the use of cryogenic probes. Shown in Figure 1 are 1H–15N TROSY spectra of the human visual arrestin protein, which binds to light-activated...
which are now known to occur when arrestins are activated upon activation of arrestins to initiate noncanonical signaling pathways, the partially disordered state represents the structural change underlying resulting in extensive exchange line broadening (and peak disappearance) following the formation of a complex with unlabeled P-Rh*.

Recent collaboration between the Wu and Stevens laboratories utilized 19F NMR to probe biased signaling pathways of the β2-adrenergic receptor (β2AR), a GPCR. This work built on earlier 13C NMR studies of this same system by the Kobilka lab. Binding of various agonists to the β2AR induces a conformational change in the receptor that activates G-protein association on the cytosolic face of the receptor. The G-protein binding site includes the cytosolic end of transmembrane segment 6 (TM6). While agonists also induce changes in structure that impact the cytosolic end of TM7 and the membrane proximal region of the adjacent C-terminus, these segments are thought not to be directly involved in G-protein binding. Instead, agonists stimulate phosphorylation of juxtamembrane residues of the C-terminus that results in activation of association with β-arrestin, which can block G-protein association, leading to endocytosis and downregulation of the receptor. Induced changes in the structure of the bound β-arrestin can also activate various pathways of signal transduction in the cell. It is known that certain agonists stimulate G-protein-based signaling versus β-arrestin-based effects to different degrees, with those agonists that preferentially stimulate β-arrestin binding and/or signaling being known as “biased agonists”.

Solution NMR studies of larger proteins have traditionally been limited to only those proteins that can be generated in uniformly 15N- and 13C-labeled forms.

While a number of recent advances are based on using NMR methods of formidable complexity, simple 1D solution NMR methods can be used to address highly significant biological questions. A variation of this approach involves fluorine NMR. 19F is a spin-1/2 nucleus with an NMR sensitivity that approaches that of 31P at a 2:1 ratio. Moreover, 19F NMR is a spin-1/2 nucleus with an NMR sensitivity that approaches that of 31P at a 2:1 ratio. Moreover, 19F NMR chemical shifts are highly sensitive to local environment, such that 19F NMR generally yields well-resolved 1D NMR spectra, making 19F an excellent reporter probe in binding studies or in studies of protein folding or conformational changes.

Recent collaborative work between the Wu and Stevens laboratories utilized 19F NMR to probe biased signaling pathways of the β2-adrenergic receptor (β2AR), a GPCR. This work built on earlier 13C NMR studies of this same system by the Kobilka lab. Binding of various agonists to the β2AR induces a conformational change in the receptor that activates G-protein association on the cytosolic face of the receptor. The G-protein binding site includes the cytosolic end of transmembrane segment 6 (TM6). While agonists also induce changes in structure that impact the cytosolic end of TM7 and the membrane proximal region of the adjacent C-terminus, these segments are thought not to be directly involved in G-protein binding. Instead, agonists stimulate phosphorylation of juxtamembrane residues of the C-terminus that results in activation of association with β-arrestin, which can block G-protein association, leading to endocytosis and downregulation of the receptor. Induced changes in the structure of the bound β-arrestin can also activate various pathways of signal transduction in the cell. It is known that certain agonists stimulate G-protein-based signaling versus β-arrestin-based effects to different degrees, with those agonists that preferentially stimulate β-arrestin binding and/or signaling being known as “biased agonists”.

To probe the underlying structural biology of biased agonism, certain β2AR cysteines (C265, C327, and C341) were labeled with a 19F-containing thiol-reactive reagent (Figure 2A). C265 and C327 are located at the cytoplasmic ends of TM6 and TM7, respectively. C341 served as a control site, being located on the membrane-distal C-terminus where its local environment is insensitive to the β2AR signaling state. 200 μL samples were used, containing 10–20 μM β2AR in mixed micelles. The 19F NMR spectra of both C265 and C327 of the unstimulated receptor each exhibited a pair of peaks that are believed to correspond to slowly interconverting activated and inactive states (Figure 2B). Not surprisingly, classical agonists shifted the population of the peaks for 19F probes at both C265 (TM6) and C327 (TM7) to increase the active-inactive ratio (Figure 2B). Biased agonists induced a shift of the inactive-to-active 19F peaks for C327 (at TM7) greater than that observed for C265. Remarkably, it can be seen that the ratios of the two states as judged by the peaks from 19F at C265 versus C327 are, for most compounds, not equal. These results suggest that there are at least two functional states: inactive, G-protein coupling-activated (reflected by a change in chemical shift for 19F at C265), and β-arrestin binding-activated (reflected by a change in the chemical shift at C327). Different compounds alter the relative populations between these states in distinct ways. This exemplifies the profound insight into protein dynamics and function that can sometimes be gleaned from even the most simple of NMR measurements.

Shimada and co-workers used 13C to address the nature of "partial agonism", whereby G-protein-coupled receptors are activated by compounds that stimulate a level of signaling that is significantly lower ("reduced efficacy") than that induced by "full agonists". For their work, β2AR was expressed in insect cells using a culture supplemented with 13CH3-methionine. 2D 1H–13C HSQC spectra of S–40 μM receptor in its ligand-free state were compared to spectra acquired when the receptor was saturated by an inverse agonist, a neutral antagonist, a pair of partial agonists, or a full agonist. These spectra revealed that saturation of β2AR by partial agonists tipped the equilibrium between the active state and a pair of inactive states toward the active state, but not to the extent of full agonists. No support was generated in this study for the notion that partial agonists result in a lower efficacy because they convert the receptor into an active state that is both structurally and functionally distinct from the active state generated by full agonists.

In related work, Kobilka and co-workers used a nearly identical NMR approach in studies of β2AR but focused on a somewhat different mechanistic question. Their work showed a difference in the nature of the conformational state induced...
by binding of a full agonist relative to the case in which the receptor was saturated with both a full agonist and a nanobody that mimics binding of β2AR’s cognate G-protein (Gs). They interpreted their data to indicate that full activation occurs only in the presence of both the agonist and the Gs mimic. When saturated by the agonist alone, the receptor appears to be in a conformationally heterogeneous state that is distinct from the fully activated state. It may be this former state that is stabilized to various degrees by various partial agonists in the Shimada study.36

THE USE OF NMR TO MONITOR PROTEIN−LIGAND AND PROTEIN−PROTEIN INTERACTIONS HAS BEEN EXTENDED

NMR spectroscopy has long been used to characterize protein−ligand or protein−protein interactions,38−40 being well-suited for studies of weak binding (Kd in the micro- to millimolar range) where the lifetime of the complex formed is too short to isolate the complex via pull-down or other methods. The simplest and most commonly used method is to monitor changes in protein 1H−15N HSQC (or TROSY) resonance positions induced by titration of a ligand or another protein. This “differential chemical shift perturbation method” is based on the notion that NMR-active nuclei located at the binding interface usually undergo larger changes in chemical shift than resonances for distal sites. In ideal cases, it is possible to map the location of the binding site on the protein based on shift perturbation patterns and to determine binding affinity by monitoring the ligand concentration dependence of observed changes in peak positions.

Our lab recently demonstrated how monitoring chemical shift perturbation can be an effective means of investigating the interaction between membrane protein C99 and cholesterol. C99 is the single-transmembrane span C-terminal domain of the amyloid precursor protein (APP) that is released by β-secretase cleavage of the full-length APP. Cleavage of C99 by γ-secretase releases the amyloid-β polypeptides that are closely related to the etiology of Alzheimer’s disease. Elevated levels of cholesterol promote amyloid-β production. While the mechanism for this effect is not well understood, it is thought that both β- and γ-secretase preferentially associate with cholesterol-rich membrane domains often termed “lipid rafts”.41 Structural studies of C99 in detergent micelles42,43 led us to hypothesize that this protein might have a cholesterol binding site. Following early studies in micelles that employed a watersoluble cholesterol analogue,42 we switched to the use of isotropic lipid/detergent bicelles, which can solubilize bona fide cholesterol up to a concentration of 20 mol % (one cholesterol for every four molecules of phospholipid and detergent). NMR experiments were used to monitor C99 in bicelles upon titration with cholesterol, revealing that it forms a 1:1 complex with C99 characterized by a Kd of 5 mol %,43 a value well within the physiological range of cholesterol in mammalian membranes. In addition to determining Kd, site-specific chemical shift perturbations mapped the location of the binding pocket, subsequently verified by mutagenesis studies (Figure 3A). The discovery that C99 is a cholesterol binding protein led the hypothesis that this binding event results in partitioning of C99 into lipid rafts, where the amyloidogenic β- and γ-secretase are preferentially localized,43 thereby activating amyloid-β production. This offers a compelling explanation for how elevated cholesterol levels promote amyloidogenesis.

We have also used NMR to illuminate the mechanisms by which a family of potential anti-Alzheimer’s drugs, known as

Figure 3. (A) Binding of cholesterol to the C99 domain of the amyloid precursor protein. Binding is believed to involve docking of the flat and rigid cholesterol to the flat surface provided by tandem GXXXG motifs on the surface of C99’s upper transmembrane helix. Formation of hydrogen bonds between the hydroxyl headgroup of cholesterol and C99 involves a conformational change (or conformational selection) centering on a flexible loop that connects a short surface-associated helix and C99’s transmembrane domain. (B) Distinct classes of interaction between two transmembrane proteins that can now be distinguished using NMR methods. Panel B taken with permission from ref 51. Copyright 2011 American Chemical Society.
γ-secretase modulators (GSMs), exert their potentially therapeutic effect, namely to selectively and favorably alter the γ-secretase production ratio between less toxic short forms of amyloid-β (such as Aβ40) and much more toxic long forms (such as Aβ42). While it has been proposed that GSMs act by first forming a complex with C99 prior to association with γ-secretase, a series of NMR experiments failed to yield any evidence that GSMs form a complex with monomeric or dimeric forms of C99 in micelles or model membranes, an observation now supported by a number of biochemical studies from other laboratories.

In addition to monitoring protein–small molecule interactions, NMR spectroscopy can monitor protein–protein interactions. We recently conducted a study of whether transmembrane C99 can form a complex in model membranes with another single-span membrane protein, CD147, representative of a difficult problem in membrane biophysics. Through mechanisms that are not well understood, CD147 is believed to reduce levels of amyloid-β in cell cultures. In our studies, a simple set of experiments were developed to classify intermolecular interactions as either (i) a specific and stoichiometric association, (ii) a nonspecific association, or (iii) “forced co-habitation” whereby two molecules are forced to interact by virtue of being entrapped in the same model membrane unit (i.e., within the same micelle) (Figure 3B). These methods allowed us to establish that CD147 associates more avidly with monomeric C99 than C99 self-associates to form homodimers. Heterodimerization between C99 and CD147 does appear to be structurally specific but is weak and may not be physiologically relevant. A final and groundbreaking example of using NMR to study a membrane protein complex is determination by the laboratories of Ulmer and Qin of the structure of the heterodimeric transmembrane domain of platelet integrin αβ3 in and an organic solvent mixture, respectively.

### NMR Paramagnetic Relaxation Enhancement (PRE) Extends the Capabilities of NMR to Probe Protein Structure and Dynamics

Early in the development of NMR, the physics of nuclear relaxation enhancement arising from the presence of proximal paramagnetic species was explored. NMR signals are broadened to a degree that is proportional to 1/T1, where r is the distance between the paramagnet and the NMR nucleus. This broadening stems from the “paramagnetic relaxation enhancement” (PRE) phenomenon. In biological NMR, paramagnets can be added to samples as free probes [such as Mn(II) and Gd(III) and their chelates] or can be attached to proteins either through metal ion coordination or by modifying free cysteines with thiol-reactive nitroxide spin-labeled compounds such as MTSL [S-(2,5,5-tetramethyl-2,5-dihydro-1H-pyrro1-3-yl)methyl methanethiosulfonate]. PRE of NMR sites can be quantitated using methods as simple as recording 1D or 2D NMR spectra of matched samples, one containing a paramagnet and the other in which the paramagnet is absent or quenched to a diamagnetic form. The PRE effect can be detected over a broad range from approximately 0 to 25 Å for a proton experiencing relaxation enhancement from a nitroxide spin-label. The distance can be extended to approximately 35 Å if a more powerful paramagnetic species is used (e.g., Mn²⁺).

The most common PRE measurements involve use of 2D 1H–15N HSQC or TROSY to measure the distances between a nitroxide spin-label fixed at a specific protein site and the backbone amide protons of the same protein, an experiment that can generate dozens of distances from a single pair of NMR spectra. The derived distances can be used as a source of restraints for structure determination. Such measurements have proven to be especially important for structural studies of challenging molecules in which only the backbone chemical shift assignments are available, as is commonly the case for integral membrane proteins.

Most recently, the use of the PRE effect has been extended to aid in the NMR assignment process, increase the sensitivity of NMR experiments, serve as a route for mapping intermolecular binding surfaces of macromolecular complexes, elucidate the active and/or binding sites of proteins, map the topology of membrane proteins, and probe dynamic and sparsely populated states of macromolecules.

α-Synuclein is under intense study because of its propensity to aggregate and form Parkinson’s disease-related fibrils. For many proteins involved in aggregation-based diseases such as Parkinson’s and Alzheimer’s, the mechanism of protein fibril formation is currently not well understood. The Baum group recently utilized PRE-based experiments to probe the interchain interactions of the intrinsically disordered protein α-synuclein, leading to a new hypothesis for its mechanism of amyloid formation. α-Synuclein has an uneven distribution of charged amino acids across its sequence. NMR-based PRE experiments were used to show that the transient encounter complexes of α-synuclein have a nonrandom distribution and that the properties of the transient encounter complex are pH-dependent. Under acidic conditions, α-synuclein is estimated to be relatively charge-neutral and was shown to favor relatively strong parallel (tail-to-tail) interchain interactions, factors that may explain why fibril formation is faster at acidic pH values. On the other hand, at more neutral pH values, α-synuclein is more highly charged and was shown to heteroassociate only weakly and with an antiparallel (head-to-tail) interaction. The results from these PRE studies suggest both a potential mechanism for the increased kinetics of α-synuclein fibrillation occurring at low pH (as in endosomes) and a qualitative mechanism for how the normally soluble protein may be rendered insoluble. This study highlights the high sensitivity of PREs to transient interactions and how they can provide insight into intrinsically disordered proteins that is hard to obtain using other methods.

Clore and co-workers have been instrumental in the exploration of sparsely populated macromolecular states with PREs. Tang et al. probed the apo and sugar-bound holo state of the maltose binding protein (MBP) with PREs and compared the results to existing X-ray crystallography structures of these states. The authors found that PRE measurements for sugar-bound MBP were consistent with the crystal structure of the protein–carbohydrate complex (Figure 4A). On the other hand, the PRE data for apo-MBP were characteristic of a rapidly exchanging mixture between an open state (captured in the apo-MBD crystal structure) and a rare and transient (~3% population) structure (Figure 4B). The rare partially closed apo-MBP was characterized using the PRE data and shown to be a partially closed state that is distinct from either the open or closed (holo) crystal structures but more closely resembles the conformation of the maltose-complexed holoprotein. It is likely that it is to this rare excited state that maltose initially binds, allowing induced fit to the stable holo form. These data expressly support the existence of a dynamic equilibrium that samples conformational space beyond the bound and apo structural forms of MBP and shows that carbohydrate binding results in an induced-fit complex.
**METHYL TROSY NMR ALLOWS NMR TO PROBE STRUCTURAL AND MECHANISTIC QUESTIONS FOR >200 kDa PROTEIN COMPLEXES**

The $^1$H–$^{13}$C NMR signals from protein side chain methyl groups provide a powerful approach to probing the structures, dynamics, and interactions of very large proteins or complexes, often providing a route to useful data under conditions where others (such as $^1$H–$^{15}$N TROSY) fail.\(^\text{79}–\text{81}\) This stems from (i) the favorable relaxation properties and motions associated with side chain methyl $^1$H and $^{13}$C, (ii) the more favorable gyromagnetic ratio of $^{13}$C compared to that of $^{15}$N, (iii) the fact that there are three protons per methyl group, and (iv) the existence of favorable (slow) relaxation pathways that can be spectroscopically selected for using the classic HMQC pulse sequence originally developed in the late 1970s.\(^\text{82}–\text{84}\) These properties have led to the HMQC-based “methyl-TROSY” family of multidimensional $^1$H–$^{13}$C NMR experiments.

To take maximal advantage of methyl-TROSY-based approaches, it is necessary to label proteins with $^{13}$C and preserve methyl protons for selected amino acids within a protein while at the same time perdeuterating all other hydrocarbon sites.\(^\text{85}–\text{87}\) Along with these labeling methods, a series of multidimensional NMR experiments have been developed that allow side chain methyl groups to be assigned on the basis of the correlation of their signals with already assigned backbone resonances.\(^\text{88}–\text{90}\) For very large proteins in which backbone resonance assignments may not be available or feasible, mutagenesis can sometimes be used as a route to assign methyl peaks.\(^\text{91}–\text{93}\)

Methyl-TROSY methods have been applied to large proteins and complexes, such as malate synthase G (81.4 kDa),\(^\text{94}\) aspartate transcarbamoylase (306 kDa),\(^\text{95}\) SecA (204 kDa),\(^\text{96}\) p53 tetramer–DNA complex (210 kDa),\(^\text{97}\) and sensory

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**Figure 4.** NMR-observed (red) and crystal structure back-calculated (black) PRE values for the maltose binding protein (MBP) in the holo (maltose-associated) (A) and apo (B) states. Red bars on the top of the plots indicate regions where signal intensities are broadened beyond detection as a result of relaxation enhancements. The holo-MBP PRE values show excellent agreement with the crystal structure, indicating that the maltose-bound holo (closed) state protein is relatively rigid when complexed with maltose under both NMR and crystal conditions. The discrepancies in panel B between the observed and back-calculated PRE values for the apo state indicate a rapidly exchanging mixture of a pair of structural states. These were determined to be the open state (similar to the apo crystal structure) and a 5% partially closed conformer. This latter structure is distinct from both the apo and holo state crystal structures but may represent the excited state conformer that initially binds maltose, which then induces a transition to the stable holo state. The inset in panel B is a surface representation of MBP with the green surface highlighting the regions where observed and back-calculated PREs do not agree. The electrostatic surface of the open state of apo MBP (C) highlights the sugar binding pocket, with panel D illustrating the differences in the MBP CTD between the partially closed apo-MBP (green cylinders) and closed holo-MBP states (red cylinders). Thus, the data in this figure show the utility of the PRE to detect and probe minor protein populations that are challenging to observe with other techniques. In this case, the minor population observed in the apo state is thought to be critical for ligand recognition and induced-fit transition to the stable holo maltose–MBP conformation. This figure is a composite from those in ref 63 and used by permission of the publisher. Copyright 2007 Nature Publishing Group.
rhodopsin in micelles (70 kDa protein–detergent complex).\textsuperscript{98} Besides providing a route to structure determination, methyl-TROSY-based data can provide powerful insight into protein–protein interactions,\textsuperscript{99} protein–ligand interactions, protein dynamics,\textsuperscript{91} and protein–DNA interaction.\textsuperscript{86}

In perhaps the most impressive example, Sprangers and Kay successfully applied methyl-TROSY methods to the 670 kDa 20S proteasome core particle, which consists of four heptameric rings arranged as $\alpha_7\beta_7\beta_7$.\textsuperscript{91} They were able to assign the $^1$H–$^13$C resonances for $\sim$90 of 97 ILV methyl groups present in the $\alpha$ subunits of this complex. A set of residues within the antechamber that separates the entrance from the catalytic site were seen to undergo correlated millisecond motion that may be related to threading of the substrate into the proteolytic site. These studies also suggested that the 12 N-terminal amino acids can exchange between the outside of the protein assembly and the lumen of the antechamber, likely acting as an access gate (Figure 5).\textsuperscript{91} This was confirmed in a follow-up study in which the $\alpha$ subunit was labeled at its N-terminus with methyl-$^1$H/$^13$C-methionine. The methionine methyl-labeled $\alpha$ subunit was also spin-labeled at various sites, and PREs were measured to yield spin-label–methionine distances, which confirmed that the N-terminus does indeed populate slowly exchanging (time scale of seconds) outside and lumenal configurations. Additional experiments established that when all seven $\alpha$ N-termini are in the outside configuration, the proteasome is fully active, whereas activity is reduced when N-termini extend into the lumen of the proteasome, occluding access of the substrate to the active site.\textsuperscript{100} Studies were also conducted of binding of the 11S activator to the 670 kDa $\alpha_7\beta_7\beta_7$ species to form a 1100 kDa $\alpha_7\beta_7\beta_7$–11S complex that mapped the 11S binding sites on the $\alpha_7$ rings and also provided a measurement of binding affinity.\textsuperscript{91}

Methyl-TROSY has also been successfully applied in membrane protein structure determination. Phototaxis receptor sensory rhodopsin II (pSRII) from Natronomonas pharaonis, a seven-transmembrane helix blue light phototactic receptor, functions via trans–cis isomerization of its retinal group, allowing its host to seek the dark when respiratory substrates are plentiful. As a membrane protein, Ile, Leu, and Val residues account for 32% of the pSRII sequence and are distributed throughout all seven helices. Using methyl-TROSY-based NMR, Nietlispach and co-workers successfully assigned numerous aliphatic methyl $^1$H and $^13$C resonances and went on to measure a large number of distance restraints based on methyl–methyl and methyl–amide $^1$H–$^1$H NOEs.\textsuperscript{98} This led to the determination of the pSRII structure, which shows an root-mean-square deviation for the backbone residues (1–221) of only 0.48 Å and is in excellent agreement with the crystal structure. Methyl-TROSY methods have also been applied in studies of other multispan transmembrane proteins, including VDAC-1,\textsuperscript{101,102} KcsA,\textsuperscript{103} and KpOmpA.\textsuperscript{104}

**INCREASED USE OF NMR IN DRUG DISCOVERY**

The 2010 Pharma R&D Annual Review reported more than 9000 drug candidates in various phases of the drug development
pipeline (http://www.pharmaprojects.com/therapy_analysis/annual-review-2010.htm). However, despite the vast resources being devoted to drug development, only 30 novel new drugs were approved by the Food and Drug Administration in 2011. Moreover, the average cost of bringing a drug to the stage of approval approaches $1 billion. An important early stage of drug development is lead compound discovery, a process that usually involves screening of large compound libraries. NMR methods have been developed for high-throughput screening of compounds directed toward validated drug targets as a route to identifying leads.

Perhaps the greatest impact of NMR in drug discovery has been the seminal role this method played in establishing an approach to lead compound development now usually termed “fragment-based drug/lead discovery” (FBDD or FBLD).\(^\text{105}\) The first practical implementation of this approach was dubbed “SAR by NMR” by Fesik and co-workers.\(^\text{106,107}\) The original NMR-centric form of this approach requires that the structure of the target protein and its NMR resonance assignments be known. By monitoring protein resonances, NMR is used to identify lead molecules that bind to spatially adjacent sites (often with only very modest efficiency), followed by the synthesis of second-generation molecules in which two lead molecules are chemically tethered to each other with a linker of rationally chosen length. This generates a new lead compound whose affinity reflects the partial additivity of the binding energies of the original unlinked pair of ligands. (Figure 6A).

The FBDD approach is being vigorously pursued in many drug discovery laboratories (sometimes using NMR, sometimes not) and has been used to develop at least one approved drug and 26 compounds in various stages of clinical trials.\(^\text{108,109}\) ABT-263 (Navitoclax, in phase II clinical trials) is an inhibitor of the anti-apoptotic (pro-survival) proteins Bcl-2 and Bcl-X\(_L\), and represents the outcome of NMR-based discovery of two lead compounds that bind to the hydrophobic BH3-binding region of Bcl-X\(_L\) (Figure 6B). 4′-Fluoro-biphenyl-4-carboxylic acid and 5,6,7,8-tetrahydronaphthalene were found by NMR to bind with modest affinity to different but proximal sites in a functionally significant domain of Bcl-X\(_L\) (\(K_d\) values of 0.3 and 4 mM, respectively). Covalently linking the two lead compounds resulted in dramatically increased binding affinity for Bcl-X\(_L\) (\(K_i = 36\) nM). This was followed by more traditional medicinal chemical optimization to generate the final ABT-263, which has a \(K_i\) of \(\leq 1\) nM toward both Bcl-2 and Bcl-X\(_L\).\(^\text{110,111}\)

NVP-AUY922 is an inhibitor of the ATPase activity of the chaperone Hsp90 and is currently in phase II clinical trials for

Figure 6. (A) Illustration of fragment-based drug design (FBDD) and both protein- and ligand-detected screening. The binding of small molecule fragments to a protein target can be detected by NMR even when the affinity is low (100 \(\mu\)M to 10 mM). High-affinity ligands can be created by linking together low-affinity fragments that bind to adjacent sites. In the protein-detected mode, peaks from nuclei located at the binding interface shift when a candidate molecular fragment binds, an approach that has the advantage of suggesting the location of the binding site in the target. In the ligand-detected mode, small molecules that bind to a target protein are identified on the basis of peak shifts or peak broadening or disappearance as a result of binding. (B) Chemical evolution of ABT-263.
the treatment of cancer. This compound was developed using FBDD relying on a second class of NMR screening methods based on detection of the signal from the candidate ligands being screened against a target (Figure 6A). Ligand-detected NMR screening can employ a variety of readouts, including simple observation of ligand peak disappearance (indicating tight binding to a very large or even insoluble target), peak shifts or line broadening, magnetization transfer from a possibly NMR-invisible target to a ligand, or observation of a change in the translational diffusion coefficient of a molecule. In the development of NVP-AUY922, more than 1000 fragments were initially tested for binding to 10−7 M Hsp90 using ligand-based NMR screening experiments, resulting in a number of resorcinol- or phenol-containing candidate fragments. Subsequent determination of the crystal structure of the complexes between the fragments and Hsp90, followed by fragment linkage and further optimization, resulted in NVP-AUY922 M112,113

An interesting recent variation of ligand-detected NMR screening is target-immobilized NMR screening (TINS), which has proven to be suitable for fragment-based drug discovery targeting membrane proteins. In TINS, a target and a negative control reference protein (both bathed in model membranes such as nanodiscs) are immobilized on a solid support in separate flow-through NMR tubes. The tubes are then loaded into a dual-cell, flow injection sample holder located within a special NMR probe.114 The mixture of compounds to be screened is simultaneously injected into both tubes. 1D 1H NMR spectra of the small molecule mixture from both NMR tubes are obtained by performing space-selective spectroscopy.115 If a small molecule binds to the immobilized target, its peak amplitude decreases (relative to the reference sample) because the peak from the bound state became undetectably broad because of association with the immobilized target. It is possible to repeatedly use the same immobilized membrane protein sample to screen an entire fragment collection (>1000 compounds), such that only ~25 nmol of membrane protein may be required to complete an initial screen (1 mg for a 40 kDa protein).116

NMR CAN NOW BE USED TO PROBE PROTEIN STRUCTURE AND INTERACTIONS IN LIVING CELLS

Recently, successful experiments for studying proteins under truly physiological conditions have been reported.117,118 “In-cell” NMR spectroscopy utilizes isotopically labeled proteins to selectively observe the protein of interest over numerous other cellular components. Various protein labeling and/or delivery methodologies have been developed. One method is the delivery of isotopically labeled recombinant proteins into oocytes using microinjection of up to ~20 nL per egg.119−122 This method allows direct control of the concentration of the delivered protein. Another approach for introducing proteins into eukaryotic cells is to tag the protein of interest with a “cell-penetrating peptide” (CPP) sequence. This allows the transfer of the protein from the extracellular medium to the cell interior via penetration of the cellular membrane.122−124 Pore-forming toxins are used as CPP.125 Another approach for introducing labeled protein into living cells is known as “single-protein production” (SPP). SPP is based on first growing E. coli in unlabeled medium, followed by triggering degradation of all the mRNA in the cells except for that encoding the target protein. The cells are then transferred to a medium containing NMR-active isotopes, and protein expression is induced, resulting in isotopic labeling of the target protein in a cellular background of unlabeled proteins.126 Expression of isotopically labeled proteins in yeast and subsequent collection of NMR spectra of these proteins in situ have recently been reported.127

The first structure of a protein determined in live cells was reported by Ito and co-workers.128,129 The putative heavy metal binding protein TTHA1718 was expressed in E. coli using the SPP method. The intact cells containing labeled TTHA1718 were then transferred to NMR tubes, followed by resonance assignments and structure determination. Rapid data collection using nonlinear sampling and selective protonation of aliphatic methyl groups (in otherwise deuterated protein) was crucial for the success of this effort. The structure determined in cells was similar to that of the purified protein, although modest differences were seen in the conformation of solvent-exposed loops.

In the case of eukaryotic cells, TROSY-based NMR spectra of reasonably high quality have been obtained from labeled protein that was microinjected into Xenopus oocytes. Selenkow et al.119 compared the NMR spectra of the recombinant [15N]GB1 domain in isolation, in oocyte cell extracts, and injected inside oocytes, revealing similar spectra. Chemical shifts of this protein in oocytes were very similar to those of purified protein, but some of the peaks were distorted or split, indicating some structural heterogeneity generated by the cellular milieu.

The ability of NMR to study the specific interactions of drugs with proteins in cells has also been documented. The interaction of the immunosuppressants FK-506 and rapamycin with the protein FKBP12 has been observed using in-cell NMR.122 15N-labeled FKBP12 was introduced into HeLa cells using a CPP construct. Changes in its 1H−15N HSQC NMR spectra were observed when FK506 or rapamycin was administered to the cell, which were similar to the changes induced when purified FKBP12 was titrated with these compounds. This work serves as a prototype for future studies of protein−ligand interactions using NMR spectroscopy of mammalian cells.

In-cell NMR remains in its infancy but clearly provides a powerful approach to studying proteins and their interactions in a truly native environment. Interesting applications are already beginning to be reported. For example, the Pielak lab has reported the surprising observation that the crowded cell cytosol does not provide stabilization of a marginally stable protein relative to its stability as a pure protein in standard buffer solutions.130 In another example, Shekhtman and co-workers expressed the FKBP and FRB proteins in E. coli.131 In higher organisms, these proteins form a ternary complex with rapamycin that leads to cell cycle arrest. NMR was then used to screen a 289-member dipeptide library to discover compounds that could both permeate the cells and mimic rapamycin by forming a ternary complex with FKBP and FRB. One of the dipeptides identified through this screen was then shown to be able to inhibit growth of yeast in a rapamycin-like manner by interacting with the yeast homologues to FKBP and FRB.132

CELL FREE EXPRESSION METHODS EXPAND THE APPLICABILITY AND EFFICIENCY OF NMR

Cell free (CF) expression systems are based on the use of extracted or purified transcription and translation machinery in cellular extracts from wheat germ or E. coli.132−135 The most important impact of these methods on NMR spectroscopy has been to facilitate isotopic labeling schemes that are impossible or impractical using cellular expression methods.136−140
Access to these advanced labeling methods can be critical to tackling difficult problems in protein structure and function.

One of the common hurdles to backbone resonance assignments for helical MPs is that their transmembrane sequences are typically dominated by only six types of amino acids, which can result in near degeneracy in the spin connectivity patterns of the multidimensional NMR data sets used to make resonance assignments. To overcome this obstacle, an optimized combinatorial dual-isotope labeling method has been developed in conjunction with CF expression to facilitate rapid resonance assignments. In this method, each sample is prepared using a subset of site-selectively carbonyl $^{13}$C-labeled and amide $^{15}$N-labeled amino acids using CF expression. The backbone residues can then be identified according to peak patterns in HSQC and HNCO spectra. Software (MCCL; http://sbl.salk.edu/combipro) is available to optimize the labeling schemes and to minimize both the number of required samples and the complexity of the spectra. The combination of this approach resulted in determination of the backbone structures of six additional human integral membrane proteins. More recently, this approach resulted in determination of the backbone structures of six additional human integral membrane proteins.

Work from the Dötsch lab shows how CF expression can be used to further enhance the study of MPs when roadblocks are reached with traditional methods. Proteorhodopsin is a retinal-binding heptahelical membrane protein that functions as a proton pump in a marine bacterium. Although almost all backbone NH resonances of proteorhodopsin were detected under diheptanonylphosphatidylcholine micelle conditions, this protein still yielded poorly resolved NMR spectra, making resonance assignments difficult and hindering measurements of NOE-derived distance restraints. Employment of the SAIL (stereo-array isotope labeling) method that uses synthetic stereoisotopically labeled amino acids in combination with CF expression provided significantly simplified spectra and facilitated partial completion of side chain assignments for residues in the transmembrane domain. Together with measured long-range NOEs, PREs, and RDCs, the generated assignments determined the first ever proteorhodopsin structure.

**RELAXATION DISPERSION PROVIDES UNPRECEDENTED ACCESS TO CRYPTIC STRUCTURAL AND DYNAMIC STATES**

Many biomacromolecules are intrinsically flexible, and characterizing their dynamics is the key to understanding their mechanisms of function in diverse biological processes. NMR is unparalleled as a tool for studying dynamics because it can provide atomic-resolution information about motions occurring over all time scales.

Among the most important recent developments in NMR has been the widespread application of relaxation dispersion experiments that can simultaneously probe both structure and dynamics. In relaxation dispersion experiments, contributions to the NMR spin–spin relaxation time ($T_2$) from conformational transitions occurring on the micro- to millisecond time scale are modulated as a function of the frequency of the field generated by Carr–Purcell–Meiboom–Gill (CPMG) refocusing or spin-lock pulse trains. Information about these conformational transitions can be determined from plots of $T_2$ versus field strength for each peak. In cases where two (and sometimes more) conformations are in equilibrium, relaxation dispersion experiments can provide the exchange rate, the relative populations, and the chemical shift differences between exchanging states of the molecule. In favorable cases, it is also possible to measure residual dipolar coupling and residual chemical shift anisotropies for each state. Such data can illuminate the kinetics of the transitions between states, the equilibrium constant, and the structures of the molecule in each state, even when one is only marginally populated. NMR relaxation dispersion methods have been widely used in studies of RNA and proteins.

Relaxation dispersion methods have contributed tremendously to the recent recognition of how dynamics critically impact enzyme mechanisms, including the development of the concepts of “dynamic coupling” of steps along reaction pathways, and “conformational selection” by ligands of dynamically sampled excited states that correspond to the structure of the protein in the complex to be formed. A classic example is provided by studies of dihydrofolate reductase (DHFR) by the laboratories of Wright, Dyson, Lee, and others. DHFR catalyzes the reduction of 7,8-dihydrofolate (DHF) to 7,8-tetrahydrofolate (THF) by the cofactor NADPH and is a key enzyme in the folate biosynthetic pathway that is important for cell growth and proliferation. DHFR’s reaction pathway contains five distinct complexes with different combinations of bound substrates and products (see Figure 7). Relaxation dispersion NMR studies have shown that DHFR dynamics on the micro- to millisecond time scale are closely coupled to the progression of its catalytic pathway. It has been observed that for each complex along the reaction cycle, the enzyme dynamically samples “excited” states that have
structures and their propensities to aggregate \(^{154,155}\) are illustrated calculated on the basis of these data. Native and intermediate state dispersion data. The structure of the intermediate was then mutant Fyn SH3 domain were extracted from a set of relaxation residual dipolar couplings of this folding intermediate state of the chemical shift anisotropies, and the backbone amide \(^{15}N\) conditions of the NMR experiments, this intermediate is

Another pioneering application of NMR relaxation dispersion is the study of the intermediates in protein folding and aggregation. Accessing the structures of these intermediates has always been difficult, with NMR-based amide H−D exchange rate measurements being perhaps the most powerful of the classical approaches for probing the structures of these states.\(^{153}\) Relaxation dispersion methods have recently taken studies of folding intermediates to a higher level of insight. The Kay group determined the structure of a folding intermediate for a mutant form of the Fyn SH3 domain using NMR relaxation dispersion spectroscopy.\(^{154}\) Under the equilibrium conditions of the NMR experiments, this intermediate is populated at only 2%. The backbone chemical shift assignments \(^{1}H_{\alpha}, \; ^{1}H_{\beta}, \; ^{15}N, \; ^{13}CO, \; ^{13}C_{\alpha}, \; \text{and} \; ^{13}C_{\beta}\), the \(^{13}CO\) residual chemical shift anisotropies, and the backbone amide \(^{15}N−^{1}H\) residual dipolar couplings of this folding intermediate state of the mutant Fyn SH3 domain were extracted from a set of relaxation dispersion data. The structure of the intermediate was then calculated on the basis of these data. Native and intermediate state structures and their propensities to aggregate\(^ {154,155}\) are illustrated in Figure 8. In the folded ground state, the mutant Fyn SH3 domain shows an incomplete \(\beta\) barrel structure with five \(\beta\) strands, among which the C-terminal \(\beta5\) strand undergoes hydrogen bonding with the N-terminal \(\beta1\) strand. In the folding intermediate state, the C-terminus (including the residues that comprise \(\beta5\)) is disordered, which potentially makes the \(\beta1\) strand available as a template for aggregation via formation of inappropriate intermolecular \(\beta\) sheet formation. However, this normally occurs only rarely because of the short lifetime and low population of this intermediate state. The role of the C-terminal \(\beta5\) strand in the long-lived folded state therefore seems to be to prevent the formation of the aggregates. This was confirmed by the finding that removal of four residues from the C-terminus prevents the formation of strand \(\beta5\) and leads to spontaneous formation of fibrillar aggregates. This work illustrates both how folding rates are tuned so that aggregation is avoided in an otherwise susceptible folding intermediate state and how the conformation of the folded state has been optimized to include structural elements that play roles to suppress aggregation.

### NMR HAS PROVIDED SEMINAL INSIGHT INTO INTRINSICALLY DISORDERED PROTEINS AND TETHERED MULTIDOMAIN PROTEINS

NMR spectroscopy has proven to be a powerful tool for characterizing intrinsically disordered proteins (IDPs), partly because of its ability to interrogate protein structure and dynamics at both very local (residue-specific) and global scales.\(^ {156}\) The finding that many peptide hormones normally adopt fully or partially disordered conformations was demonstrated by \(^{1}H\) NMR spectroscopy in the early 1970s.\(^ {157}\) However, the fact that numerous larger proteins are intrinsically disordered long escaped recognition, reflecting the implicit assumption that disordered proteins must represent non-native and nonfunctional forms. Seminal work in the 1990s\(^ {158,159}\) led to the realization that many proteins are designed by nature to be intrinsically disordered in a way that is closely linked to native function and intermolecular interactions.\(^ {160−163}\) The fact that these domains and proteins often have very specific functions and propensities to form ordered structures upon complex formation with proteins or other molecules is now a well-established paradigm in molecular biology.\(^ {164}\)

Because of its ability to inform on both macromolecular conformations and dynamics, NMR has been the primary technique used to discover and study IDPs, both in their free state and after complex formation. It has been revealed that IDPs and proteins with natively disordered domains play a dazzling number of biological roles and that their conformational behavior is rich and varied. For example, it is now appreciated that even complex formation between two proteins may not reflect only a single mode of interaction but may instead represent a composite of exchanging binding modes, each of which in isolation is of only low affinity, but which sum
as a dynamic equilibrium to yield a much higher overall affinity. Forman-Kay, Mittag, and co-workers have shown this to be the case for formation of the complex between the multi-phosphorylated Sic1 protein (a cyclin-dependent kinase inhibitor) and the WD40 domain of the Cdc4 component of the SCF<sup>Cdc4</sup> ubiquitin ligase (Figure 9).<sup>165−167</sup> There is only a single primary phosphate binding site in Cdc4/WD40, and it has been shown that this site can engage serially with different phosphates on Sic1, with the unliganded phosphates possibly contributing additional electrostatic stabilization. The resulting complex is dynamic both in the sense that the composite affinity between Sic1 and Cdc4 reflects the partial summation of the affinities for the various exchanging binding modes and because Sic1 remains largely disordered even after the formation of the complex with Cdc4. By altering the extent and patterns of phosphorylation, cell biology may be able to tune binding affinity and specificity to achieve a dynamic range and complexity of binding and/or response behavior that extends far beyond what is possible with simple two-state regulation.\(^{168}\) It seems likely that there are other entirely new paradigms involving protein disorder and biological complexity waiting to be discovered.\(^{169}\) The importance of IDPs in biology and the role that NMR has played in discovering and characterizing these proteins are difficult to overstate.

In the case of multidomain proteins, disordered regions between domains facilitate modular domain behavior and interdomain flexibility. High-quality NMR spectra can often be obtained even for very large multidomain proteins because of tether-enabled domain motions. For example, in studies of replication protein A, Chazin and co-workers used NMR to pinpoint the sequential locations of tethered and folded domains and to characterize the dynamics and relative orientations of the tethered domains.\(^{170}\) Veenhoff and her co-workers used 1D $^1$H spectra and 2D $^1$H−$^{13}$C HSQC spectra in their investigation of the pathway traveled by the Heh2 integral membrane protein from the outer nuclear membrane to reach the inner nuclear membrane.\(^{171}\) This study revealed that Heh2 remains membrane-associated and diffuses from one membrane domain to the other through the linking membrane that lines the orifice of the nuclear pore complex. The transmembrane domain of Heh2 is connected to membrane-distal domains that include its nuclear localization sequences (NLS) by a 150-residue linker (L) domain. Simple NMR methods were used to show that the L domain is an intrinsically disordered protein. The L domain appears to play a critical role in the transport of Heh2 through the nuclear pore by providing a flexible tether to the membrane-distal domains that allows these domains to clear the core scaffold of the pores and to be recognized by trafficking partner proteins. L domain flexibility also allows these distal Heh2 domains and their associated partner proteins to bob and weave as they associate with successive FG-Nup proteins in the process of making their way through the junglelike matrix of the nuclear pore.

**CONCLUSIONS**

We have surveyed technical advances that, in concert with progress in sample preparation, are transforming the applicability of NMR to proteins. This has led to recent NMR application to problems of great significance, such as studies of GPCR and proteasome function—structure relationships. Other applications have thus far been prototypical in nature, such as most applications of NMR to specific proteins in living cells. Other emerging methods not covered in this review, such as nonclassical ways of collecting and processing pulsed NMR data,\(^{172−174}\) can also be expected to further expand the problem-solving capability of solution NMR, particularly if such approaches can be widely implemented in user-friendly form. While soothsaying is perilous, all indicators point to a bright future for NMR as a tool for studying protein structure, folding, dynamics, interactions, and function.

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